

## Online Supplement

### Exposure to a high fat diet alters leptin sensitivity and elevates renal sympathetic nerve activity and arterial pressure in rabbits

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## **Materials and Methods**

### **Dietary information**

The control diet was standard rabbit chow (SF06-011, Specialty Feeds, Glen Forrest, Australia) containing 2.63 kcal/g, of which 4.2% of calories were from fat, 18.2% protein, 14.1% crude fiber and 18% acid detergent fiber. The HFD was modified standard rabbit chow with 5% pork fat (lard) and 5% soya oil (SF02-005, Specialty Feeds). This diet contained 3.34 kcal/g, consisting of 13.3% calories from fat, 17.5% protein, 13.1% crude fiber, 16% acid detergent fiber. Rabbits on a control diet were meal fed 100g standard rabbit chow per day plus 30g of chaff whilst those on HFD were given ad libitum access to HFD pellets. All received ¼ of an apple per day. Differences in macronutrient and fatty acid composition of the HFD compared to the normal diet is shown in Fig S1.

### **Measurement of cardiovascular variables and renal sympathetic nerve activity**

Mean arterial pressure (MAP), heart rate (HR) and renal sympathetic nerve activity (RSNA) were measured in conscious rabbits. On the day of the experiment, the animal was placed in a standard single rabbit holding box (length, width, height; 40, 14, 21 cm) with wire top and raised wire grid floor.

MAP and HR were measured from the central ear artery catheterized transcutaneously with a 22-gauge catheter (BD Insyte, Singapore) connected to a pressure transducer (Statham P23DG transducer, Hato Rey, Puerto Rico) via polypropylene tubing filled with heparinised saline (12.5 IU/ml) to prevent clotting. The pulsatile arterial pressure from the transducer was amplified (D.C. amp NT218, Neomedix, NSW, Australia) and digitized by an acquisition card (PC Plus, National Instruments, Austin, TX, USA) using software written in the LabView programming language (National Instruments) by Dr. Elena Lukoshkova. MAP (mmHg), was calculated on a beat-to-beat basis and instantaneous HR (bpm) was calculated from the pulse interval. Zero pressure was taken at the rabbit's mid-chest level, and the pressure transducer was calibrated to 100 mmHg against a mercury sphygmomanometer prior to the start of each experiment.

RSNA was measured via a renal nerve electrode implanted at least 6-7 days prior to experimentation. The electrode plug was connected via a cable to a low noise preamplifier and amplifier combination model (Baker IDI Heart and Diabetes Institute Models 187b and 190). Postganglionic RSNA was recorded with a bandwidth of 50 Hz to 2 kHz and amplified potentials were rectified and integrated using an integrator filtered with a 20-millisecond time constant. Integrated RSNA was digitized and averaged over two-second periods using the Labview program. As well as integrated total RSNA, Labview also detected and recorded the frequency of bursts of synchronised sympathetic activity and the maximum amplitude of each burst as described previously<sup>1</sup>.

The RSNA was measured in microvolts ( $\mu$ V). However, there was a wide variation in the raw nerve activity between rabbits, which was most likely due to the different physical conditions of the implanted electrode.<sup>2</sup> Thus, amplitude and total RSNA value was normalised to its maximal nerve output that was elicited by the nasopharyngeal reflex.<sup>3</sup> This was evoked by exposing the rabbit to 50 ml of cigarette smoke, which was collected in a syringe and released into the rabbit's box. The maximum of the largest sympathetic burst during the response was recorded and taken to equal 100 normalised units (nu) and all RSNA was expressed in terms of this. This procedure was done at the beginning of the experimental day.

### **c-Fos Immunohistochemistry**

Rabbits were deeply anaesthetized with an intraperitoneal injection of sodium pentobarbitone (100 mg/kg) 90 minutes after the administration of leptin (100 µg in 50 µl, i.c.v.). The animals were perfused transcardially with 1L of 0.9 % saline and 1L of 4 % paraformaldehyde dissolved in 0.1 M phosphate buffer, pH 7.2 (PB). Subsequently, the brain was removed and postfixed for 3 hours in 20 % sucrose in paraformaldehyde, and placed in 20 % sucrose in PB and refrigerated overnight at approximately 4 °C. Coronal sections (40 µm) were cut on a cryostat and placed in PB. Free-floating sections were incubated in 10 % normal horse serum at room temperature for 1 hour. Sections from control and HFD rabbits were processed in tandem being incubated with the same dilution of antibody and then aliquoted into separate vials. Sections were incubated in primary antibody, goat anti-c-Fos (Santa Cruz) diluted 1:100 in a solution of 2 % normal horse serum and 0.3 % Triton X-100 (Sigma) in PB at room temperature overnight. Sections were washed in PB prior to incubation in biotinylated donkey anti-goat immunoglobins (1:200, Jackson) in PB containing 2 % normal horse serum for 1 hour. Thereafter, the sections were washed and incubated in avidin-biotin peroxidase complex (1:100, Vector) in PB for 1 hour. Following washes in 0.05 M Tris buffer (pH 7.6), sections were incubated in a solution of 40 mg nickel ammonium sulphate and 50 mg 3-3' diaminobenzidine hydrochloride per 100 ml Tris buffer for 10 min, 15 µl of 30 % hydrogen peroxide was added for a further 6 min. Following final washes sections were mounted on gelatine coated microscope slides.

### **c-Fos Immunohistochemical Analysis**

Bright-field illumination using a Motic BA400 microscope and Motic images plus 2.0 were used to assess sections that exhibited c-Fos-immunoreactivity as detected by black stained nuclei. Brain sites examined include the medial preoptic nucleus (MPO), median preoptic nucleus (MnPO), periventricular paraventricular nucleus of the hypothalamus (pPVN), rostral PVN (rPVN), supraoptic nucleus (SON), arcuate nucleus (ARC), dorsomedial hypothalamus (DMH) and ventromedial hypothalamus (VMH) as defined by Girgis and Shih-Chang rabbit brain atlas.<sup>4</sup> A blind analysis of up to three sections per animal for each brain site and counts of total black stained nuclei within the known boundaries of the nucleus were recorded.

### **Plasma leptin assay**

Arterial blood was drawn into chilled tubes containing EGTA. Blood samples were then centrifuged and plasma was stored at -80°C until assay. Plasma leptin levels were measured in duplicate by radioimmunoassay using a multi-species leptin kit (LINCO Research, St. Charles, USA), according to the manufacturer's instructions.

## References

1. Malpas SC, Bendle RD, Head GA, Ricketts JH. Frequency and amplitude of sympathetic discharges by baroreflexes during hypoxia in conscious rabbits. *Am J Physiol.* 1996;271:H2563-H2574.
2. Dorward PK, Riedel W, Burke SL, Gipps J, Korner PI. The renal sympathetic baroreflex in the rabbit. Arterial and cardiac baroreceptor influences, resetting, and effect of anesthesia. *Circ Res.* 1985;57:618-633.
3. Burke SL, Head GA. Method for in-vivo calibration of renal sympathetic nerve activity in rabbits. *J Neurosci Methods.* 2003;127:63-74.
4. Girgis M, Shih-Chang W: *A new stereotaxic atlas of the rabbit brain.* St Louis, Missouri, USA, Warren H. Green, Inc., 1981.

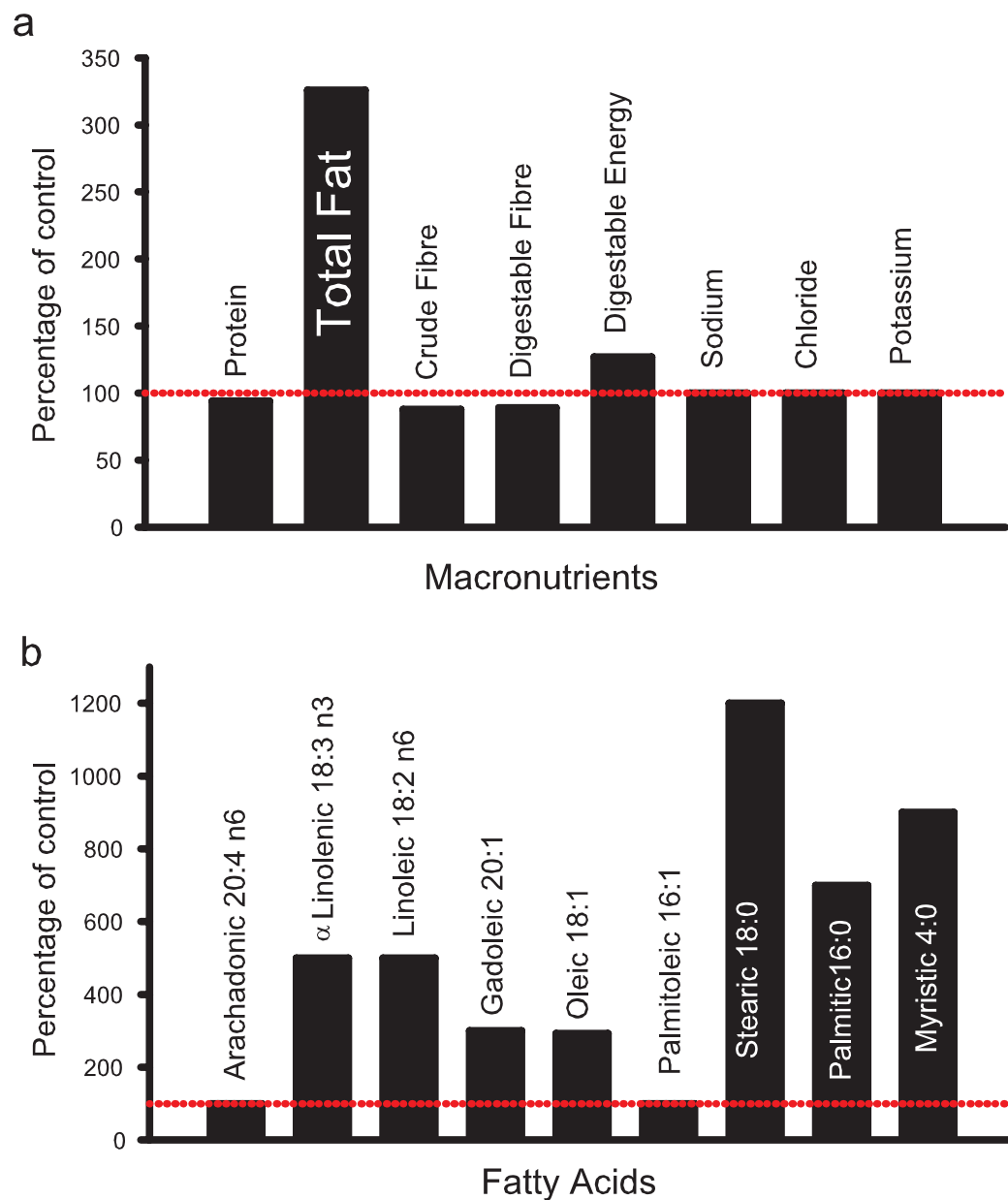


Figure S1: Comparison of dietary nutrients (**a**) and fatty acid (**b**) analysis from the normal fat control diet and high fat diet (HFD).

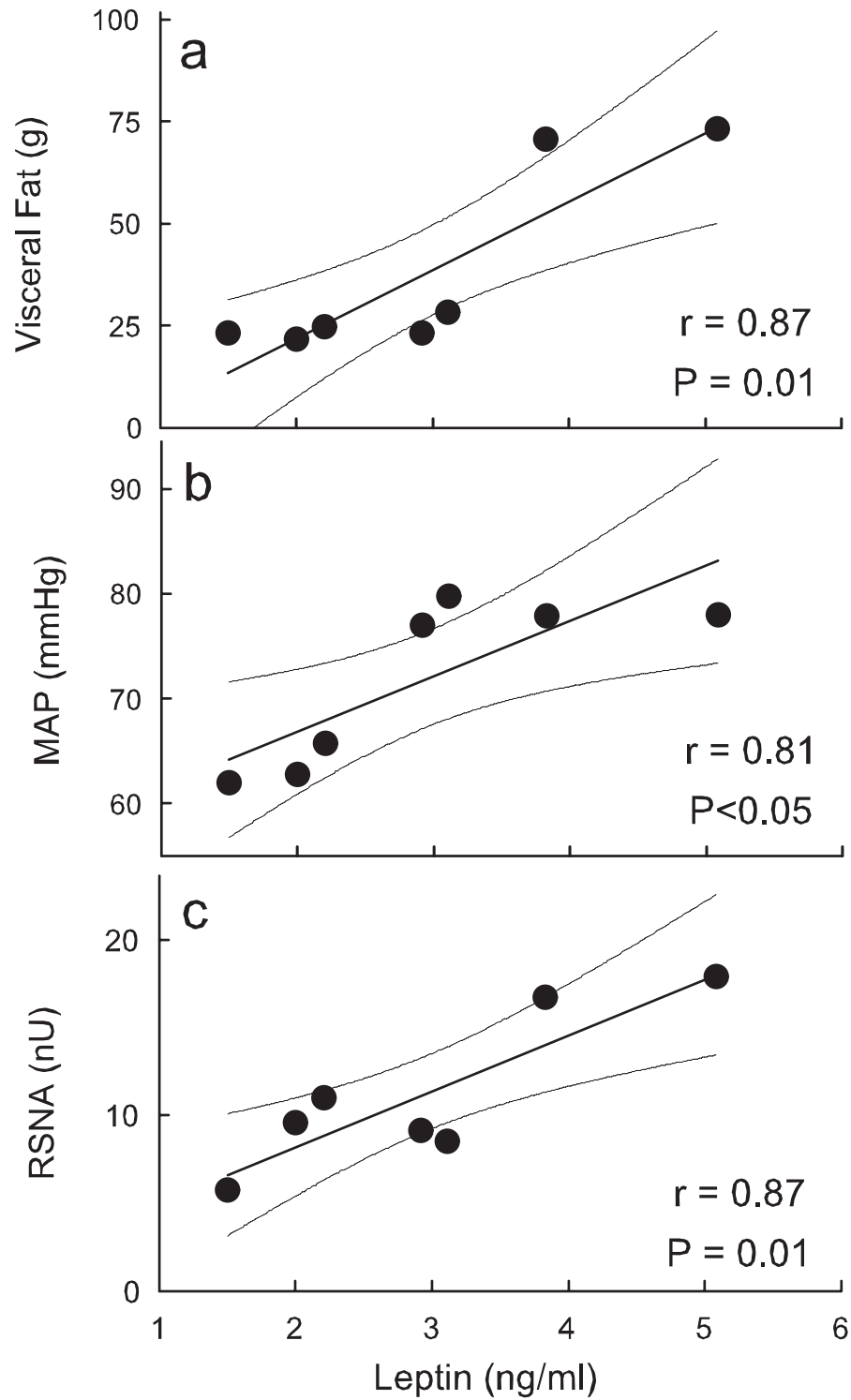


Figure S2 Correlation analysis of plasma leptin (ng/ml) to (a) Visceral WAT (g), (b) mean arterial pressure (MAP, mmHg) and (c) renal sympathetic nerve activity (RSNA, nu) in 7 rabbits fed either a normal or High fat diet. The correlation was calculated using a least squares regression (thick line), with 95 % confidence intervals (thin line) and  $r$  is the correlation coefficient.

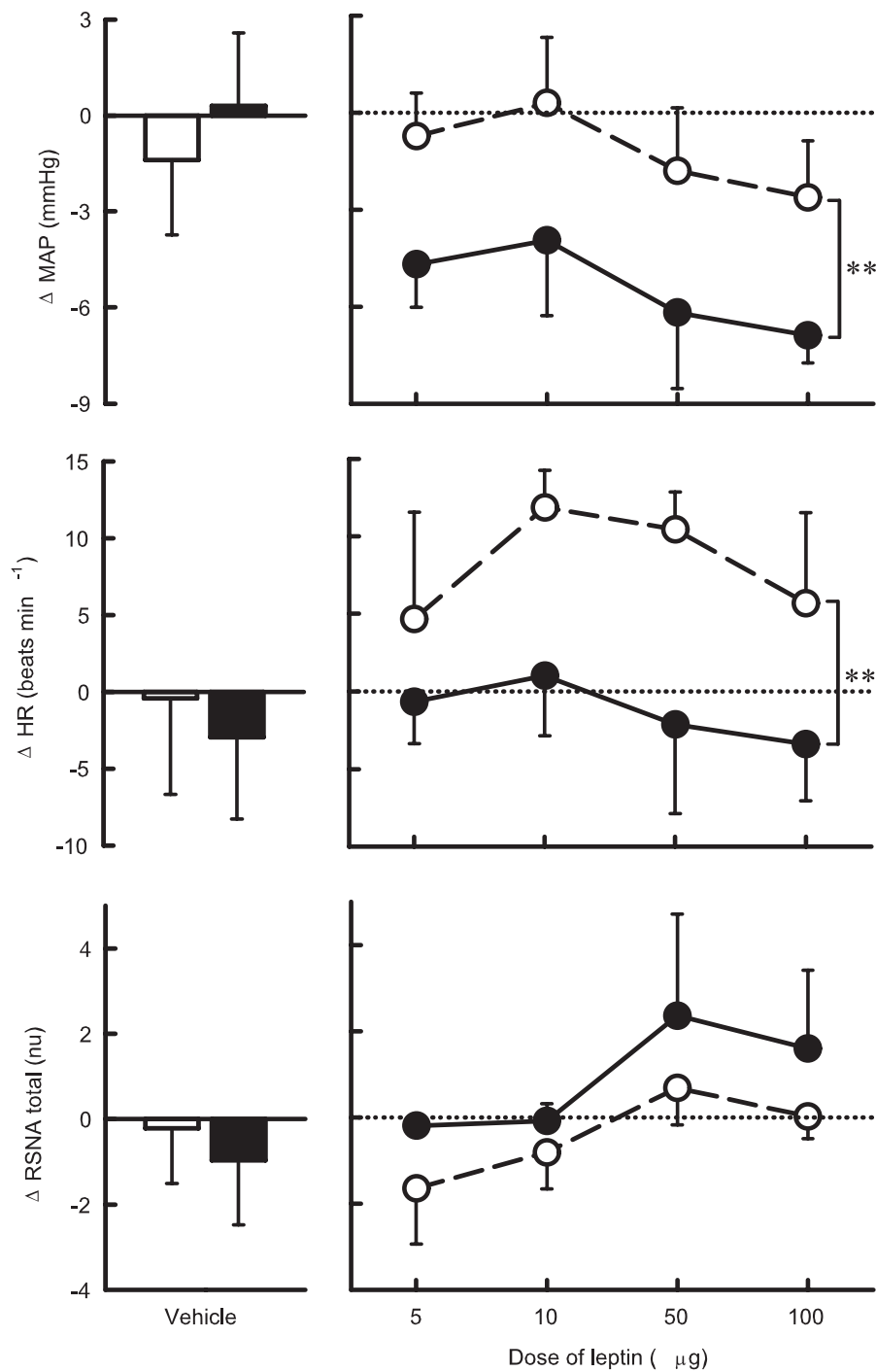


Figure S3: Left panels: Changes from baseline in mean arterial pressure (MAP, upper), heart rate (HR, middle) and renal sympathetic nerve activity (RSNA, lower) following intravenous (iv) administration of vehicle (saline) in rabbits fed a control diet (open bars, n = 4) or high fat diet (HFD, closed bars, n = 5) for three weeks. Right panels: Thirty-minute averages of the changes in MAP (upper), HR (middle) and RSNA (lower) from vehicle (in left panel) following i.v. administration of leptin to control diet rabbits (open circles) and HFD (closed circles) rabbits. RSNA is expressed as total RSNA in normalized units (nu). Error bars are SEM indicating variance between animals. \*\*P < 0.01 for comparison between groups.

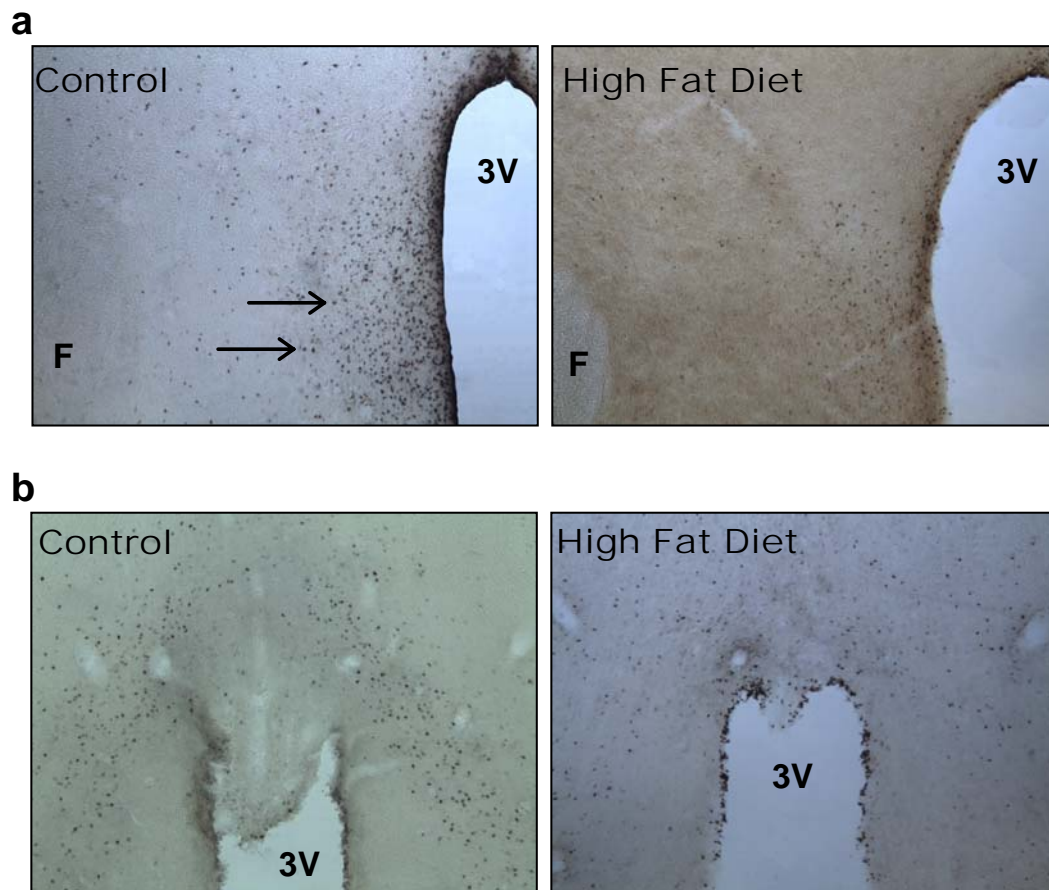


Figure S4: Representative photomicrographs of coronal sections through the rostral paraventricular nucleus (rPVN, **a**) and medial preoptic nucleus (MPO, **b**) showing c-Fos labeled neurons (as indicated by the arrows) following an intracerebroventricular infusion of leptin (100 $\mu$ g) in rabbits fed a normal fat diet or high fat diet for four weeks. Abbreviations: 3V, third ventricles; F, fornix.